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(54) Title: PROCESS OF OBTAINING COMPOSITIONS ENRICHED FOR HEMATOPOIETIC STEM CELLS AND ANTIBODIES FOR USE THEREIN

(57) Abstract

This invention provides methods to obtain compositions substantially enriched for hematopoietic stem cells. Compositions obtained by the practice of these methods also are provided. The methods employ a separation regimen utilizing antibodies that specifically recognize and bind a novel hematopoietic stem cell marker designated herein as EM16. Monoclonal antibodies to this marker as well as the cell lines that produce these monoclonal antibodies are further provided.

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PROCESS OF OBTAINING COMPOSITIONS ENRICHED FOR HEMATOPOIETIC STEM CELLS AND ANTIBODIES FOR USE THEREIN

The field of this invention is the isolation of a population of cells enriched for human hematopoietic stem cells.

Mammalian hematopoietic cells provide a diverse range of physiological activities. These cells are divided into lymphoid, myeloid and erythroid lineages. The lymphoid lineage, comprising B cells and T cells, provides for the production of antibodies, regulation of the cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. The myeloid lineage, which includes monocytes, granulocytes, megakaryocytes as well as other cells, monitors for the presence of foreign bodies, provides protection against neoplastic cells, scavenges foreign materials, produces platelets, and the like. The erythroid lineage provides the red blood cells, which act as oxygen carriers.

Despite the diversity of the nature, morphology, characteristics and function of hematopoietic cells, it is presently believed that these cells are derived from a single precursor cell population, termed "stem cells." Stem cells are capable of self-regeneration and can become lineage committed progenitors which are dedicated to differentiation and expansion into a specific lineage. As used herein, "stem cells" refers to hematopoietic cells and not stem cells of other cell types.

A pluripotent stem cell can be defined by possessing the following characteristics: (1) gives rise to progeny in all defined hematolymphoid lineages; and (2) a limiting numbers of cells are capable of fully reconstituting a seriously immunocompromised host in all blood cell types and their progenitors, including the pluripotent hematopoietic stem cell, by self-renewal.

A highly purified population of stem cells is necessary for a variety of *in vitro* experiments and *in vivo* indications. For instance, a purified population of stem cells will allow for identification of growth factors associated with their self-regeneration. In addition, there can be as yet undiscovered growth factors associated with: (1) the

early steps of dedication of the stem cell to a particular lineage; (2) the prevention of such dedication; and (3) the positive or negative control of stem cell proliferation.

Stem cells find use in: (1) regenerating the hematopoietic system of a host deficient in any class of hematopoietic cells; (2) a host that is diseased and can be treated by removal of bone marrow, isolation of stem cells and treatment with drugs or irradiation prior to re-engraftment of stem cells; (3) producing various hematopoietic cells; (4) detecting and evaluating growth factors relevant to stem cell self-regeneration; and (5) the development of hematopoietic cell lineages and assaying for factors associated with hematopoietic development.

Stem cells also are important targets for gene therapy, where the inserted genes promote the health of the individual into whom the stem cells are transplanted. In addition, the ability to isolate stem cells can serve in the treatment of lymphomas and leukemias, as well as other neoplastic conditions where the stem cells are purified from tumor cells in the bone marrow or peripheral blood, and reinfused into a patient after myelosuppressive or myeloablative chemotherapy. Thus, there have been world-wide efforts toward isolating stem cells in substantially pure or pure form.

Stem cells constitute only a small percentage of the total number of hematopoietic cells. Hematopoietic cells are identifiable by the presence of a variety of cell surface "markers." Such markers can be either specific to a particular lineage or progenitor cell or can be present on more than one cell type. Currently, it is not known how many of the markers associated with differentiated cells are also present on stem cells. One marker, which was previously indicated as present solely on stem cells, CD34, is also found on a significant number of lineage committed progenitors. U.S. Pat. No. 4,714,680 describes a population of cells expressing the CD34 marker.

In view of the small proportion of the total number of cells in the bone marrow or peripheral blood which are stem cells, the uncertainty of the markers associated with the stem cell as distinct from more differentiated cells, and the general difficulty in assaying for stem cells biologically, the identification and purification of stem cells has been elusive. Characterizations and isolation of stem cells are reported in: Baum et al. (1992) Proc. Natl. Acad. Sci. USA 89:2804-2808; and Tsukamoto et al. U.S. Patent No. 5,061,620.

Decreased rhodamine 123 (rho123) staining of hematopoietic cells appears to correlate to stem cell potential. This so-called "rho^{lo}" marker is determined not by the initial dye accumulation but by an efflux process sensitive to P-glycoprotein (P-gp) inhibitors. Retention of several P-gp-transported fluorescent dyes, including rho123, in human bone marrow cells was inversely correlated with the expression of P-gp. Bone marrow cells expressing physical and antigenic characteristics of pluripotent stem cells show high levels of P-gp expression and fluorescent dye efflux. Fractions of human bone marrow cells isolated on the basis of either increased rho123 efflux or P-gp expression contain practically all the primitive progenitor cells of human bone marrow, including long-term culture-initiating cells (LTC-IC). Chaudhary and Roninson (1991) Cell 66:85-94.

Recently, the mouse stem cell has been obtained in at least highly concentrated, if not purified form, where fewer than about 30 cells obtained from bone marrow were able to reconstitute all of the lineages of the hematopoietic system of a lethally irradiated mouse. Each assayed cell is multipotent for all hematopoietic lineages, while self-renewal is variable amongst these cells. Spangrude et al. (1988) Science 241:58-62; Smith et al. (1991) Proc. Natl. Acad. Sci. USA 88:2788-2792; Uchida (1992) Ph.D. Thesis Stanford U.; and see also, EPA 89 304651.6 and the references cited therein, which describe the isolation of mouse stem cells.

According to the present invention, methods are provided to obtain compositions substantially enriched for hematopoietic stem cells. The methods employ a separation regimen utilizing antibodies (hereinafter identified as αEM16) specific for a unique cell surface marker (hereinafter identified as EM16) that is expressed on stem and progenitor cells, while being less accessible or absent on more mature cells. Cells that express this marker are hereinafter identified as EM16^{+}.

Positive selection of stem cells with antibodies that recognize EM16 can be used in combination with selection techniques that isolate cells expressing other stem cell markers and/or techniques that isolate cells or a cell population lacking lineage-specific (LIN^{-}) markers. Enriched populations of cells derived from these methods are also provided.

Figure 1, panels A through D, shows FACS™ analysis of EM16 and rhodamine 123 staining (panel D), with isotype IgM control (panel C), of $CD34^+Thy\text{-}1^+$, viable, low side scatter gated bone marrow cells (panels A and B).

Figure 2, panel A through E, shows FACS™ analysis of $CD34^+$ bone marrow cells (panel A) staining with I-CD38 and an anti-Thy-1 (panel C), with IgG1 isotype control (panel B), and staining with I-CD38 and I-EM16 (panel E), with IgM isotype control (panel D). The y-axis shows CD38 staining; the x-axis shows staining of the antibody being tested (Thy-1, EM16, IgM isotype or IgG isotype).

Figure 3, panels A through C, shows FACS analysis of $CD34^+$ bone marrow cells (control, panel A), stained with α-EM16 (y-axis) and Thy-1 (x-axis) (panel C), with IgM isotype control (panel B).

Figure 4, panels A through D, compares Thy-1 versus CD34 expression (panel B), with EM16 versus CD34 expression (panel D). Panels A & C show isotype controls. The x-axis is CD34 expression; the y-axis is the antibody being tested (Thy-1, EM16, IgM isotype or IgG isotype).

Figure 5 is a graph plotting CAFC frequency after 3 to 5 weeks in culture.

DEPOSIT INFORMATION

A hybridoma cell line producing an antibody that specifically recognizes the EM16 marker was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852 U.S.A., on May 1, 1996, under the provisions of the Budapest Treaty for the International Recognition for the Deposit of Microorganisms for the Purposes of Patent Procedure, and has been assigned accession number HB-12100.

The present invention provides methods for isolating a population of hematopoietic cells highly enriched for hematopoietic stem cells expressing, or being characterized as having the EM16 marker.

It has now been found that the cell surface marker ("EM16") recognized by antibodies specific for EM16 ("αEM16") is expressed on hematopoietic stem cells.

Antibodies to the EM16 marker bind about 50% of $CD34^+$ cells obtained from freshly ficolled ($\delta < 1.077$) cadaveric bone marrow and 3-4% of total mononuclear cells on average. As exemplified below, αEM16 has the following characteristics.

αEM16 binds 13-87%, average of 62%, of $CD34^+$ cells from mobilized peripheral blood (n=12). In 7 of 8 measurements, the binding to $CD34^-$ cells is less than or equal to 6%. In the remaining instance, binding to $CD34^-$ cells was 24% (See Table 2, below). This may be due, in part, to the variable nature of these cells for flurophor-labelled imunologic reagents.

Populations of bone marrow cells that are bound by αEM16 are characterized as follows. The Sys1 assay provided below shows that both the long (5 week) and short (3 week) term CAFC activity is enriched in the EM16 positive ($EM16^+$) subset of $CD34^+/Lin^-$ cells as compared to the EM16 negative subset ($EM16^-$). (See Table 1 and Figure 5, below). Antibodies specific to the EM16 marker, bind the majority of progenitors measured as day 14 CFU-GM and CFU-Mix, day 24 HPP, and cumulative CFU-GM (Table 1). The SCID-hu thymus assay indicates that EM16+ cells have the potential to develop into T cells, indicative of T-lymphoid potential (see Table 1). The SCID-hu bone assay indicates that $EM16^+$ cells have SCID-hu bone repopulating activity, generating B-cells and myeloid cells, indicative of primitiveness and pluripotentiality (see Table 1).

Thus, αEM16 can be used in place of antibodies specific to other stem cell markers *e.g.*, CD34, to purify the majority of progenitor cells and pluripotent stem cells from heterogeneous cell populations containing hematopoietic stem cells. Antibodies to EM16 bind to a larger subset of $CD34^+$ cells than do antibodies to Thy-1. As described more fully in the examples section, EM16 marker defines a population of cells which is enriched in CFU-GM, in addition to the CAFC activity, which provides a desirable bone marrow graft composition. Thus, the composition obtained by selection based on the EM16 marker is more rapidly regenerated and differentiated into mature lineages than a composition based on selection utilizing Thy-1. Further, based on SCID-hu thymus data, the $EM16^+CD34^+$ cell composition includes a majority of T cell progenitors and therefore, lymphoid progenitor activity.

The present invention provides methods of obtaining a composition substantially enriched in hematopoietic stem cells. As used herein, "substantially enriched" is intended to mean a population of cells, wherein greater than 65%, preferably 70%, and more preferably greater than 75%, of the cell population comprises hematopoietic stem cells characterized in being $EM16^+$.

The methods involve combining a cell population containing hematopoietic cells with an antibody that specifically recognizes and binds to EM16 under conditions which allow the antibody to specifically bind to EM16 and separating the cells recognized by the antibody. In one embodiment, the antibody is labeled with a detectable marker and after combining the cell population with the antibody, the antibody-bound cells are selected based on the presence of bound, labeled antibody on the cell or cells.

Cell populations useful in this method include, and are not limited to, cell populations obtained from bone marrow, both adult and fetal, mobilized peripheral blood (MPB) and umbilical cord blood. $EM16^+$ hematopoietic stem cells can be isolated from any known source of hematopoietic stem cells, including, but not limited to, bone marrow, both adult and fetal, mobilized peripheral blood (MPB) and umbilical cord blood. The use of umbilical cord blood is discussed, for instance, in Issaragrishi et al. (1995) N. Engl. J. Med. 332:367-369. Initially, bone marrow cells can be obtained from a source of bone marrow, including but not limited to, ilium (*e.g.*, from the hip bone via the iliac crest), tibia, femora, vertebrate, or other bone cavities. Other sources of stem cells include, but are not limited to, embryonic yolk sac, fetal liver, and fetal spleen. The methods can include further enrichment or purification procedures or steps for stem cell isolation by positive selection for other stem cell specific markers. Suitable positive stem cell markers include, but are not limited to, $CD34^+$ and $Thy\text{-}1^+$.

For isolation of bone marrow, an appropriate solution can be used to flush the bone, including, but not limited to, salt solution, conveniently supplemented with fetal calf serum (FCS) or other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from about 5-25 mM. Convenient buffers include, but are not limited to, HEPES™, phosphate buffers and lactate buffers. Otherwise bone marrow can be aspirated from the bone in accordance with conventional techniques.

This invention also provides the antibodies useful to carry out the above method and the hybridoma cell lines that produce monoclonal antibodies with the requisite specificity. For example, the invention encompasses antibodies which specifically recognize the cell surface marker having the epitope recognized by αEM16. As used herein, the term "αEM16" encompasses any antibody or fragment thereof, either native or recombinant, chimeric synthetic or naturally-derived, which retains sufficient specificity to bind specifically to the cell surface marker having the epitope recognized by αEM16. As used herein, the terms "antibody" or "antibodies" include the entire antibody and antibody fragments containing functional portions thereof. The term "antibody" includes any monospecific, bispecific or chimeric compound comprised of a sufficient portion of the light chain variable region and/or the heavy chain variable region of αEM16 to effect binding to the epitope to which the whole αEM16 antibody has binding specificity. The fragments can include the variable region of at least one heavy or light chain immunoglobulin polypeptide, and include, but are not limited to, Fab fragments, $F(ab')_2$ fragments, and Fv fragments.

In addition, the monospecific domains can be attached by any method known in the art to another suitable molecule. The attachment can be, for instance, chemical or by genetic engineering.

The αEM16 can be produced by any recombinant means known in the art. Such recombinant antibodies include, but are not limited to, fragments produced in bacteria and non-human antibodies in which the majority of the constant regions have been replaced by human antibody constant regions. In addition, such "humanized" antibodies can be obtained by host vertebrates genetically engineered to express the recombinant antibody.

As used herein, αEM16 includes the monoclonal antibody designated ATCC HB-12100, or any monoclonal antibody or polyclonal antibody, that binds specifically to EM16 in such a manner as to recognize, preferentially, hematopoietic progenitor and hematopoietic stem cells. This also includes any αEM16 having the same antigenic specificity as ATCC HB-12100. If a monoclonal or polyclonal antibody being tested specifically binds with EM16, then the antibody being tested and the antibodies provided by the hybridomas of this invention are equivalent. It also is possible to determine without undue experimentation, whether an antibody has the

same specificity as the monoclonal antibody of this invention by determining whether the antibody being tested prevents a monoclonal antibody of this invention from binding EM16. If the antibody being tested competes with a monoclonal antibody of the invention as shown by a decrease in binding by the monoclonal antibody of this invention, then it is likely that the two antibodies bind to the same or a closely related epitope. Alternatively, one can pre-incubate the monoclonal antibody of this invention with $EM16^+$ cells, and determine if the monoclonal antibody being tested is inhibited in its ability to bind the antigen. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or a closely related, epitopic specificity as the EM16 monoclonal antibody. Alternatively, if binding is not inhibited, but the antibody being tested binds all $EM16^+$ cells and shows the same staining patterns as described in the Examples herein, the antibody being tested likely binds a different epitope of the EM16 antigen and, therefore is also useful in selecting the $EM16^+$ cells of the present invention.

The EM16 antibodies or functional portions thereof are obtained by methods known in the art for production of antibodies. The actual methods used are described in the Examples presented herein although any method known in the art of antibody production can be used. Such methods include, but are not limited to, separating B cells with cell-surface antibodies of the desired specificity, cloning the DNA expressing the variable regions of the light and heavy chains and expressing the recombinant genes in a suitable host cell. Standard monoclonal antibody generation techniques can be used wherein the antibodies are obtained from immortalized antibody-producing hybridoma cells. These hybridomas can be produced by immunizing animals with stem cells, and fusing B lymphocytes from the immunized animals, preferably isolated from the immunized host spleen, with compatible immortalized cells, preferably a B cell myeloma. The resulting hybridomas are screened for their binding specificity to EM16 as described above. The hybridoma or continuous cell lines producing these antibodies are further provided by this invention.

The antibodies can be conjugated to other compounds including, but not limited to, enzymes, magnetic beads, colloidal magnetic beads, haptens, fluorochromes, metal compounds, radioactive compounds or drugs. The enzymes that can be conjugated to the antibodies include, but are not limited to, alkaline phosphatase,

peroxidase, urease and β-galactosidase. The fluorochromes that can be conjugated to the antibodies include, but are not limited to, fluorescein isothiocyanate, tetramethylrhodamine isothiocyanate, phycoerythrin, allophycocyanins and Texas Red. For additional fluorochromes that can be conjugated to antibodies see Haugland, R.P., Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals (1992-1994). The metal compounds that can be conjugated to the antibodies include, but are not limited to, ferritin, colloidal gold, and particularly, colloidal superparamagnetic beads. The haptens that can be conjugated to the antibodies include, but are not limited to, biotin, digoxigenin, oxazalone, and nitrophenol. The radioactive compounds that can be conjugated or incorporated into the antibodies are known to the art, and include but are not limited to technetium 99m (^{99}Tc), ^{125}I and amino acids comprising any radionuclides, including, but not limited to, ^{14}C, ^{3}H and ^{35}S.

Preferably, the cell population is initially subject to negative selection techniques to remove those cells that express lineage specific markers and retain those cells which are lineage negative ("LIN$^-$"). LIN$^-$ cells generally refer to cells which lack markers such as those associated with T cells (such as CD2, 3, 4 and 8), B cells (such as CD10, 19 and 20), myeloid cells (such as CD14, 15, 16 and 33), natural killer ("NK") cells (such as CD2, 16 and 56), RBC (such as glycophorin A), megakaryocytes (CD41), mast cells, eosinophils or basophils. Methods of negative selection are known in the art. The absence or low expression of such lineage specific markers is identified by the lack of binding of antibodies specific to the cell specific markers, useful in so-called "negative selection". Preferably the lineage specific markers include, but are not limited to, at least one of CD2, CD14, CD15, CD16, CD19, CD20, CD38, HLA-DR and CD71; more preferably, at least CD14 and CD15. As used herein, LIN$^-$ refers to a cell population selected based on the lack of expression of at least one lineage specific marker. A highly enriched composition can be obtained by selective isolation of cells that are CD34$^+$EM16$^+$LIN$^-$.

Various techniques can be employed to separate the cells by initially removing cells of dedicated lineage. Monoclonal antibodies are particularly useful for identifying markers associated with particular cell lineages and/or stages of differentiation. The antibodies can be attached to a solid support to allow for crude separation. The

separation techniques employed should maximize the retention of viability of the fraction to be collected. Various techniques of different efficacy can be employed to obtain "relatively crude" separations. Such separations are up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells present not having the marker can remain with the cell population to be retained. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

Procedures for separation can include, but are not limited to, physical separation, magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, *e.g.*, plate, elutriation or any other convenient technique.

The use of physical separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rho123 and DNA-binding dye Hoechst 33342). These procedures are well known to those of skill in this art.

Techniques providing accurate separation include, but are not limited to, flow cytometry, which can have varying degrees of sophistication, *e.g.*, a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, etc. Cells also can be selected by flow cytometry based on light scatter characteristics, where stem cells are selected based on low side scatter and low to medium forward scatter profiles. Cytospin preparations show the enriched stem cells to have a size between mature lymphoid cells and mature granulocytes.

Alternatively, in a first separation, typically starting with about $1 \times 10^{8-9}$, preferably at about $5 \times 10^{8-9}$ cells, αEM16 can be labeled with a first fluorochrome, while the antibodies for the various dedicated lineages, can be conjugated to a fluorochrome with different and distinguishable spectral characteristics from the first fluorochrome. While each of the lineages can be separated in more than one "separation" step, desirably the lineages are separated at the same time as one is

positively selecting for EM16 and/or other stem cell markers, e.g. ($CD34^+$, $Thy\text{-}1^+$). The cells can be selected and isolated from dead cells, by employing dyes associated with dead cells (including but not limited to, propidium iodide (PI)). Preferably, the cells are collected in a medium comprising 2% FCS.

While it is believed that the particular order of separation is not critical to this invention, the following order is preferred. The cells are initially separated by a coarse separation, e.g., density gradient centrifugation, elutriation, or chemical depletion, followed by a fine separation, e.g., magnetically stabilized fluidized bed (U.S. Patent No. 5,409,813) or flow cytometry with positive selection with αEM16.

The cells obtained as described above can be used immediately or frozen at liquid nitrogen temperatures and stored for long periods of time, being thawed and capable of being reused. The cells usually will be stored in 10% DMSO, 50% fetal calf serum (FCS), 40% RPMI 1640 medium. Once thawed, the cells can be expanded by use of growth factors and/or stromal cells associated with stem cell proliferation and differentiation.

In another embodiment of the invention, a composition highly enriched or containing a substantially purified population of stem cells is provided. The results presented herein indicate that antibodies to EM16 recognize and bind with high specificity to a cell surface antigen found on human hematopoietic cells, and exposed to a high degree on hematopoietic stem cells. This specificity can be used to isolate and purify these stem cells. Such a composition also has utility in reconstituting hematopoietic systems and in studying various parameters of hematopoietic cells.

The cells generated from $EM16^+$ cells and obtained from these cultures give rise to B cells, T cells, erythroid cells and myelomonocytic cells in the *in vivo* assays described below. *In vitro* analyses for hematopoietic progenitor cells have also been reported by Whitlock and Witte (1982) Proc. Natl. Acad. Sci. USA 79:3608-3612; and Whitlock et al. (1987) Cell 48:1009-1021.

In view of cell and tissue distribution of the EM16 marker, the finding of this marker provides the opportunity to describe molecular components specific to primitive progenitor cells. The epitope bound by EM16 is novel as determined by comparison with a large database (HILDVA V, (1993)) describing staining patterns of hundreds of antibodies to hematopoietic cells. Several cell lines as well as peripheral

blood lymphocytes (PBL) and HUVEC have been tested for the expression of the epitope bound by EM16 (See Table 3). The patterns of epitope expression observed indicate that EM16 binds to an epitope that is distinct from those bound by monoclonal antibodies to CD34,or Thy-1, and from any other antigens that have been described on hematopoietic stem cells. No binding to peripheral blood mononuclear cells is observed. The level of epitope expression is modest on both bone marrow and other positively stained cells tested. The treatment of bone marrow cells with neuraminadase or glycoprotease does not diminish the binding of these monoclonal antibodies indicating that carbohydrate components are not a major aspects of the epitope defined by the monoclonal antibodies.

The results presented herein show that the $EM16^+$ population contains all cells that are $CD34^+/Thy\text{-}1^+$, including both $rho123^{lo}$ and $rho\ 123^{hi}$ subsets. This population of cells has been shown to include the most primitive pluripotential hematopoietic stem cells. Thus, EM16 is expressed on the subset of $CD34^+$ cells that contains virtually all primitive hematopoietic stem cells and IEM16 enables the purification and isolation of this subset of hematopoietic stem cells.

Compositions having greater than 90%, usually greater than about 95% of $EM16^+$ cells can be achieved in this manner. The desired stem cells can be further enriched by selection for LIN^- and/or $CD34^+$ and/or $Thy\text{-}1^+$ and/or rho^{lo}, or combinations of the markers described herein.

By separating $CD34^+EM16^+$ cells from human hematopoietic sources, the long-term culture activity is enriched in the $EM16^+$ fraction compared to $EM16^-$. The long-term culture initiating cell (LTCIC) assay or the CAFC (cobblestone area forming cell assay) which shows a direct correlation is currently the best *in vitro* assay for stem cell activity. Moreover, the $EM16^+$ cells will generate both B and myeloid cells in long-term cultures. Taken together, these data demonstrate that the $EM16^+$ fraction contains stem cells, and selection based on EM16 expression provides an enriched population of stem cells. In further enrichments of the $EM16^+$ cells using antibodies to Thy-1 and/or any of the combinations specified in Table 3, the stem cell frequency can be further increased.

The methods can be used as a diagnostic assay for the number of stem cells in a sample of hematopoietic cells. Thus, for example, after treating a patient with

chemotherapy and/or cytokines in order to mobilize stem cells into the peripheral blood, periodic leukapheresis samples may be tested for EM16 expression, where increased EM16-positive cells in the sample indicate increased stem cell content and, therefore, optimal time to harvest blood samples having maximal stem cell content. The cells and antibody are combined under conditions sufficient to allow specific binding of the antibody to EM16 and the $EM16^+$ cells are then quantitated. In a separate embodiment, the $EM16^+$ cells can be isolated or further purified.

In vivo demonstration of sustained hematopoietic ability of the various cell populations can be accomplished by the detection of continued myeloid, erythroid and B-lymphoid cell production in the SCID-hu bone model. Kyoizumi et al. (1992) Blood 79:1704; Chen et al. (1994) Blood 84:2497. To analyze this potential, one can isolate human fetal bone and transfer a longitudinally sliced portion of this bone into the mammary fat pad of a SCID/SCID animal: the bone cavity is reduced of endogenous progenitor cells by whole body irradiation of the mouse host prior to injection of the test donor population. The HLA of the population which is injected is mismatched with the HLA of the recipient bone cells. After eight weeks, the grafts are analyzed for the presence of donor HLA^+ lymphoid and myeloid progeny. $EM16^+$ cells are found to engraft in the SCID-hu bone model, and to give rise to myeloid and lymphoid progeny.

To demonstrate differentiation to T cells, fetal thymus is isolated and cultured from 4-7 days at about 25°C, so as to deplete substantially the lymphoid population. The cells to be tested for T cell activity are then microinjected into the thymus tissue, where the HLA of the population which is injected is mismatched with the HLA of the thymus cells. The thymus tissue can then be transplanted into a scid/scid mouse as described in US Patent No. 5,147,784, particularly transplanting under the kidney capsule. Specifically, a sorted population of $EM16^+$ cells can be microinjected into HLA mismatched thymus fragments. After 6-10 weeks, assays of the thymus fragments injected with $EM16^+$ cells show that $EM16^+$ cells can generate donor-derived T cells.

In a further embodiment there is provided a composition comprising a substantially enriched population of human hematopoietic stem cells, wherein the cells express the Em16 marker.

In a yet furtherembodiment of the present ivention there is provide a composition obtainable according to the methods of the present invention.

The cell compositions obtained by the methods also can be used to fully reconstitute an immunocompromised host such as an irradiated host and/or a host subject to chemotherapy; or as a source of cells for specific lineages, by providing for their maturation, proliferation and differentiation into one or more selected lineages by employing a variety of factors, including, but not limited to, erythropoietin, colony stimulating factors, *e.g.*, GM-CSF, G-CSF, or M-CSF, interleukins, *e.g.*, IL-1, -2, -3, -4, -5, -6, -7, -8, etc., or the like, or stromal cells associated with the stem cells becoming committed to a particular lineage, or with their proliferation, maturation and differentiation.

The $EM16^+$ cells also can be used in the isolation and evaluation of factors associated with the differentiation and maturation of hematopoietic cells. Thus, the invention encompasses the use of $EM16^+$ cells in assays to determine the activity of media, such as conditioned media, or to evaluate fluids for cell growth activity, involvement with dedication of particular lineages, or the like.

The invention also encompasses treatment of diseases or amelioration of symptoms associated with disease, amenable to gene transfer into $EM16^+$ cell populations obtained by the methods disclosed herein.

A number of human genetic diseases that result from a lesion in a single gene are prime candidates for gene therapy. As used herein, the term "gene therapy" or "gene transfer" is defined as the insertion of genes into cells for the purpose of medicinal therapy. There are many applications of gene therapy, particularly via stem cell genetic insertion, and thus are well known and have been extensively reviewed.

Gene therapy using HSCs is useful to treat a genetic abnormality in lymphoid and myeloid cells that results generally in the production of a defective protein or abnormal levels of expression of the gene. For a number of these diseases, the introduction of a normal copy or functional homolog of the defective gene and the production of even small amounts of the missing gene product would have a beneficial effect. At the same time, overexpression of the gene product would not be expected to have deleterious effects. The following provides a non-exhaustive list of diseases for which gene transfer into HSCs is potentially useful. These diseases generally include bone marrow disorders, erythroid cell defects, metabolic disorders and the like. Hematopoietic stem cell gene therapy is beneficial for the treatment of genetic

disorders of blood cells such as α and β-thalassemia, sickle cell anemia and hemophilia A and B in which the globin gene or clotting factor gene is defective. Another good example is the treatment of severe combined immunodeficiency disease (SCIDS), also known as the bubble boy syndrome, in which patients lack the adenosine deaminase (ADA) enzyme which helps eliminate certain byproducts that are toxic to T and B lymphocytes and render the patients defenseless against infection. Such patients are ideal candidates to receive gene therapy by introducing the ADA gene into their HSCs instead of the patient's lymphocytes as done in the past. Other diseases include chronic granulomatosis where the neutrophils express a defective cytochrome b and Gaucher disease resulting from an abnormal glucocerebrosidase gene product in macrophages.

Strategies to treat various forms of cancer also include gene therapy. The retroviral vector can carry a gene that encodes, for eg., a toxin or an apoptosis inducer effective to specifically kill the cancerous cells. Specific killing of tumor cells can also be accomplished by introducing a suicide gene to cancerous hematopoietic cells under conditions that only the tumor cells express the suicide gene. The suicide gene product confers lethal sensitivity to the cells by converting a normally nontoxic drug to a toxic derivative. For example, the enzyme cytosine deaminase converts the nontoxic substance 5'-fluorocytosine to a toxic derivative, 5-fluorouracil (Mullen et al.(1992) Proc. natl. Acad. Sci. USA 89:33-37). Tumor-specific lymphocytes can be genetically modified for example, to locally deliver gene products with anti-tumor activity to sites of the tumor to circumvent the toxicity associated with the systemic delivery of these gene products. A gene therapy approach can also be applied to render bone marrow cells resistant to the toxic effects of chemotherapy.

Gene therapy can also be used to prevent or combat viral infections such as HIV and HTLV-1 infection. For example, HSCs can be genetically modified to render them resistant to infection by HIV. One approach is to inhibit viral gene expression specifically by using antisense RNA or by subverting existing viral regulatory pathways. Antisense RNAs complementary to retroviral RNAs have been shown to inhibit the replication of a number of retroviruses (To et al. (1986) Mol. Cell. Biol. 6:4758-4762; Hopper and Coffin (1988) In:Gluzman and Hughes (eds.), Current Communications in Biology, Viral Vectors. Cold Spring Harbor Laboratory, Cold

Spring Harbor, NY. pp 139-145) including HIV (Rhodes and James (1991) AIDS 5:145-151) and HTLV-1 (von Reuden et al. (1991) J. Virol. 63:677-682).

Another area where gene therapy in HSCs may find use is in alleviating autoimmune disease. The therapeutic gene can encode, eg., a B or T cell signalling molecule capable of reconstituting the normal apoptotic signal that results in the death and elimination of autoreactive cells.

Diseases other than those associated with hematopoietic cells can also be treated by genetic modification, where the disease is related to the lack of a particular secreted product including, but not limited to, hormones, enzymes, interferons, growth factors, or the like. By employing an appropriate regulatory initiation region, inducible production of the deficient protein can be achieved, so that production of the protein will parallel natural production, even though production will be in a different cell type from the cell type that normally produces such protein. It is also possible to insert a ribozyme, antisense or other message to inhibit particular gene products or susceptibility to diseases, particularly hematolymphotropic diseases.

The therapeutic gene is transduced into the cell by any number of methods, e.g., using adenoviral vectors, adeno-associated viral vectors, and liposomes. Adenoviral, vaccinia, canarypox viral, cationic liposomes and plasmids are useful to achieve transient expression. Preferably, retroviral vectors such as, Moloney Murine Leukemia Virus (MoMLV), Myeloproliferative Sarcoma Virus (MPSV), Murine Embryonic Stem Cell Virus (MESV) and adenovirus, are useful to achieve stable and sustained expression of the transferred gene or gene product. Direct physical methods also are available. These methods include the use of the "gene gun" or calcium phosphate transfection method.

As noted above, any method of gene transfer is encompassed by this invention. As used herein, "viral vector" is defined as a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either *in vivo*, *ex vivo* or *in vitro*. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide comprising the retroviral genome or part thereof, and a therapeutic gene. As used herein, "retroviral mediated gene transfer" or "retroviral transduction" carries the same meaning and refers to the process by which a gene or nucleic acid sequences are stably transferred into the host cell by virtue of the virus entering the cell and integrating its genome into the host cell genome. The virus

can enter the host cell via its normal mechanism of infection or be modified such that it binds to a different host cell surface receptor or ligand to enter the cell. As used herein, retroviral vector refers to a viral particle capable of introducing exogenous nucleic acid into a cell through a viral or viral-like entry mechanism.

Retroviruses carry their genetic information in the form of RNA; however, once the virus infects a cell, the RNA is reverse-transcribed into the DNA form which integrates into the genomic DNA of the infected cell. The integrated DNA form is called a provirus.

Retroviral vectors useful in the methods of this invention are produced recombinantly by procedures already taught in the art. For example, WO 94/29438 describes the construction of retroviral packaging plasmids and packaging cell lines. As is apparent to the skilled artisan, the retroviral vectors useful in the methods of this invention are capable of infecting HSCs. The techniques used to construct vectors, and transfect and infect cells are widely practiced in the art. Examples of retroviral vectors are those derived from murine, avian or primate retroviruses. Retroviral vectors based on the Moloney (Mo) murine leukemia virus (MuLV) are the most commonly used because of the availability of retroviral variants that efficiently infect human cells. Other suitable vectors include those based on the Gibbon Ape Leukemia Virus (GALV) or HIV.

In producing retroviral vector constructs derived from the Moloney murine leukemia virus (MoMLV), in most cases, the viral gag, pol and env sequences are removed from the virus, creating room for insertion of foreign DNA sequences. Genes encoded by the foreign DNA are usually expressed under the control of the strong viral promoter in the LTR. Such a construct can be packed into viral particles efficiently if the gag, pol and env functions are provided in trans by a packaging cell line. Thus, when the vector construct is introduced into the packaging cell, the gag-pol and env proteins produced by the cell, assemble with the vector RNA to produce infectious virions that are secreted into the culture medium. The virus thus produced can infect and integrate into the DNA of the target cell, but does not produce infectious viral particles since it is lacking essential packaging sequences. Most of the packaging cell lines currently in use have been transfected with separate plasmids, each containing one of the necessary coding sequences, so that multiple recombination events are necessary before a replication competent virus can be produced. Alternatively, the packaging cell

line harbors an integrated provirus. The provirus has been crippled so that, although it produces all the proteins required to assemble infectious viruses, its own RNA cannot be packaged into virus. Instead, RNA produced from the recombinant virus is packaged. The virus stock released from the packaging cells thus contains only recombinant virus.

The range of host cells that may be infected by a retrovirus or retroviral vector is determined by the viral envelope protein. The recombinant virus can be used to infect virtually any other cell type recognized by the env protein provided by the packaging cell, resulting in the integration of the viral genome in the transduced cell and the stable production of the foreign gene product. In general, murine ecotropic env of MoMLV allows infection of rodent cells, whereas amphotropic env allows infection of rodent, avian and some primate cells, including human cells. Amphotropic packaging cell lines for use with MoMLV systems are known in the art and commercially available and include, but are not limited to, PA12 and PA317. Miller et al. (1985) Mol. Cell. Biol. 5:431-437; Miller et al. (1986) Mol. Cell. Biol. 6:2895-2902; and Danos et al. (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464. Xenotropic vector systems exist which also allow infection of human cells.

The host range of retroviral vectors has been altered by substituting the env protein of the base virus with that of a second virus. The resulting, "pseudotyped", virus has the host range of the virus donating the envelope protein and expressed by the packaging cell line. Recently, the G-glycoprotein from vesicular stomatitis virus (VSV-G) has been substituted for the MoMLV env protein. Burns et al. (1993) Proc. Natl. Acad. Sci USA 90:8033-8037; and PCT patent application WO 92/14829. Since infection is not dependent on a specific receptor, VSV-G pseudotyped vectors have a broad host range.

Usually, the vectors will contain at least two heterologous genes or gene sequences: (i) the therapeutic gene to be transferred; and (ii) a marker gene that enables tracking of infected cells. As used herein, "therapeutic gene" can be an entire gene or only the functionally active fragment of the gene capable of compensating for the deficiency in the patient that arises from the defective endogenous gene. Therapeutic gene also encompasses antisense oligonucleotides or genes useful for antisense suppression and ribozymes for ribozyme-mediated therapy. Therapeutic genes that encode dominant inhibitory oligonucleotides and peptides as well as genes

that encode regulatory proteins and oligonucleotides also are encompassed by this invention. Generally, gene therapy will involve the transfer of a single therapeutic gene although more than one gene may be necessary for the treatment of particular diseases. In one embodiment, the therapeutic gene is a normal, i.e. wild-type, copy of the defective gene or a functional homolog. In a separate embodiment, the therapeutic gene is a dominant inhibiting mutant of the wild-type. More than one gene can be administered per vector or alternatively, more than one gene can be delivered using several compatible vectors. Depending on the genetic defect, the therapeutic gene can include the regulatory and untranslated sequences. For gene therapy in human patients, the therapeutic gene will generally be of human origin although genes from other closely related species that exhibit high homology and biologically identical or equivalent function in humans may be used, if the gene product does not induce an adverse immune reaction in the recipient. For example, a primate insulin gene whose gene product is capable of converting glucose to glycogen in humans would be considered a functional equivalent of the human gene. The therapeutic gene suitable for use in treatment will vary with the disease. For example, a suitable therapeutic gene for treating sickle cell anemia is a normal copy of the β-globin gene. A suitable therapeutic gene for treating SCID is the normal ADA gene.

Nucleotide sequences for the therapeutic gene will generally be known in the art or can be obtained from various sequence databases such as GenBank. The therapeutic gene itself will generally be available or can be isolated and cloned using the polymerase chain reaction PCR (Perkin-Elmer) and other standard recombinant techniques. The skilled artisan will readily recognize that any therapeutic gene can be excised as a compatible restriction fragment and placed in a vector in such a manner as to allow proper expression of the therapeutic gene in hemaotpoietic cells.

A marker gene can be included in the vector for the purpose of monitoring successful transduction and for selection of cells into which the DNA has been integrated, as against cells which have not integrated the DNA construct. Various marker genes include, but are not limited to, antibiotic resistance markers, such as resistance to G418 or hygromycin. Less conveniently, negative selection may be used, including, but not limited to, where the marker is the HSV-tk gene, which will make the cells sensitive to agents such as acyclovir and gancyclovir. Alternatively, selections

could be accomplished by employment of a stable cell surface marker to select for transgene expressing stem cells by FACS™ sorting. The NcoR (neomycin /G418 resistance) gene is commonly used but any convenient marker gene whose sequences are not already present in the recipient cell, can be used.

The viral vector can be modified to incorporate chimeric envelope proteins or nonviral membrane proteins into retroviral particles to improve particle stability and expand the host range or to permit cell type-specific targeting during infection. The production of retroviral vectors that have altered host range is taught, for example, in WO 92/14829 and WO 93/14188. Retroviral vectors that can target specific cell types *in vivo* are also taught, for example, in Kasahara et al. (1994) Science 266:1373-1376. Kasahara et al. describe the construction of a Moloney leukemia virus (MoMLV) having a chimeric envelope protein consisting of human erythropoietin (EPO) fused with the viral envelope protein. This hybrid virus shows tissue tropism for human red blood progenitor cells that bear the receptor for EPO, and is therefore useful in gene therapy of sickle cell anemia and thalassemia. Retroviral vectors capable of specifically targeting infection of HSCs are preferred for *in vivo* gene therapy.

The viral constructs can be prepared in a variety of conventional ways. Numerous vectors are now available which provide the desired features, such as long terminal repeats, marker genes, and restriction sites, which may be further modified by techniques known in the art. The constructs may encode a signal peptide sequence to ensure that genes encoding cell surface or secreted proteins are properly processed post-translationally and expressed on the cell surface if appropriate. Preferably, the foreign gene(s) is under the control of a cell specific promoter.

Expression of the transferred gene can be controlled in a variety of ways depending on the purpose of gene transfer and the desired effect. Thus, the introduced gene may be put under the control of a promoter that will cause the gene to be expressed constitutively, only under specific physiologic conditions, or in particular cell types.

The retroviral LTR (long terminal repeat) is active in most hematopoietic cells *in vivo* and will generally be relied upon for transcription of the inserted sequences and their constitutive expression (Ohashi et al. (1992) Proc. Natl. Acad. Sci. 89:11332; Correll et al. (1992) Blood 80:331). Other suitable promoters include the human cytomegalovirus (CMV) immediate early promoter and the U3 region promoter of the

Moloney Murine Sarcoma Virus (MMSV), Rous Sarcoma Virus (RSV) or Spleen Focus Forming Virus (SFFV).

Examples of promoters that may be used to cause expression of the introduced sequence in specific cell types include Granzyme A for expression in T-cells and NK cells, the CD34 promoter for expression in stem and progenitor cells, the CD8 promoter for expression in cytotoxic T-cells, and the CD11b promoter for expression in myeloid cells.

Inducible promoters may be used for gene expression under certain physiologic conditions. For example, an electrophile response element may be used to induce expression of a chemoresistance gene in response to electrophilic molecules. The therapeutic benefit may be further increased by targeting the gene product to the appropriate cellular location, for example the nucleus, by attaching the appropriate localizing sequences.

The vector construct is introduced into a packaging cell line which will generate infectious virions. Packaging cell lines capable of generating high titers of replication-defective recombinant viruses are known in the art, see for example, WO 94/29438. Viral particles are harvested from the cell supernatant and purified for *in vivo* infection using methods known in the art such as by filtration of supernatants 48 hours post transfection. The viral titer is determined by infection of a constant number of appropriate cells (depending on the retrovirus) with titrations of viral supernatants. The transduction efficiency can be assayed 48 hours later by both FACS and Southern blotting.

After viral transduction, the presence of the viral vector in the transduced stem cells or their progeny can be verified such as by PCR. PCR can be performed to detect the marker gene or other virally transduced sequences. Generally, periodic blood samples are taken and PCR conveniently performed using eg. NeoR probes if the NeoR gene is used as marker. The presence of virally transduced sequences in bone marrow cells or mature hemotopoietic cells is evidence of successful reconstitution by the transduced HSCs. PCR techniques and reagents are well known in the art, See, generally, PCR Protocols, A Guide to Methods and Applications. Innis, Gelfand, Sninsky & White, eds. (Academic Press, Inc., San Diego, 1990) and commercially available (Perkin-Elmer).

When gene transfer is performed *ex vivo*, HSCs are harvested from the bone marrow or peripheral blood of the donor and the cell population is enriched for $EM16^+$. The cell population is then infected with the retroviral vector carrying a suitable therapeutic gene.

Throughout this application, various publications, patents and published patent applications are referenced to more fully describe the state of the art to which this invention pertains. The disclosures of these publications, patents and published patent applications are hereby incorporated by reference into the present disclosure.

The following examples are offered by way of illustration and not by way of limitatio**EXAMPLES**

Cell Processing and Monoclonal Antibody Production

Immunizations: C57/Bl mice were tolerized to $CD34^-$ bone marrow cells by injection with cyclophosphamide (200 mg/kg) 24 and 48 hours after i.p. injection with 10^7 CD34-bone marrow cells. After 4 weeks, this tolerization regimen was repeated. Starting approximately 1 month later, mice were immunized i.p. with $CD34^+$ cells (4×10^6 cells/mouse) or a stem cell enriched subset of $CD34^+$ cells (*e.g.*, $CD34^+$, $CD38^-$, $0.5 - 1 \times 10^6$ cells/mouse) at 4 week intervals for a total of 3 immunizations. All immunizations were in RIBI™ adjuvant (RIBI™ Adjuvant Systems). Three days after the last immunization, the spleen was harvested to create hybridomas.

Fusion to create hybridomas: Splenocytes from immunized mice were fused to the myeloma partner P3xAg8.653 (ATCC) using standard PEG mediated fusion as described in Galfre et al. (1977) Nature 266:550–552). Hybridomas from a single spleen were plated into 19 x 96-well tissue culture plates in RPMI + 10% FCS™ containing HAT™ to kill non-hybridoma cells. Fourteen days after fusion, supernatants from the hybridoma wells containing secreted antibodies were harvested for testing.

Subcloning of hybridomas: The hybridomas secreting the EM16 mAb was subcloned three to four times by limiting dilution in the presence of Hybridoma Enhancing Supplement (Sigma) to ensure monoclonality.

Cadaveric Bone Marrow and Mobilized Peripheral Blood: Cadaveric bone marrow cell suspensions derived from multi-organ donor vertebral bodies were obtained from Northwest Tissue Center (Seattle, WA). Patient peripheral blood samples were obtained after informed consent and chemotherapeutic regimens designed to mobilize primitive hematopoietic cells into the periphery. Multiple myeloma patients were mobilized with a high dose of cyclophosphamide and GM-CSF according to standard techniques. Non-Hodgkins lymphoma (NHL) patients were mobilized with VP16 and G-CSF. Normal, healthy, volunteer donors were mobilized with G-CSF.

Cells were separated over IsoPrep (Robbins Scientific, Sunnyvale, CA) harvesting the low density mononuclear cells ($\Lambda < 1.068$ g/mL for cadaveric bone marrow, $\delta < 1.077$ g/mL for peripheral blood) and were further stained with antibodies for FACS™ and analysis.

Antibody Staining For Fluorescent Activated Cell Sorting and Analysis:

Dulbecco's modified phosphate buffered saline (Ca^{++} and Mg^{++} free) supplemented with 2% fetal bovine serum, 10 mM HEPES™, 10 U/mL heparin, and 1 mg/mL human gamma globulin (Gamimune™, Miles, Elkhart, IN) was used. Cells were incubated at 1 x 10^7/mL in EM16 hybridoma supernatant diluted 1:2 in buffer for 30 minutes on ice. Cells were washed and EM16 binding was detected by adding PE-labeled rabbit anti-mouse IgM (Zymed, 1/100 dilution) and incubating for 30 minutes on ice. Cells were washed again. Cells were resuspended to 10^7/mL in 2% normal mouse and 2% normal goat serum and incubated for 10 minutes on ice. FITC™ or sulforhodamine-conjugated anti-CD34 antibody [Tük 3, $F(ab')_2$] was added at 3 Tg/mL, and to amplify the PE signal, PE-labeled goat anti-rabbit IgG was added (Zymed, 1/100) and incubated 30 minutes on ice. Cells were washed and resuspended for cell sorting or analysis in buffer containing 1 Tg/mL propidium iodide to stain non-viable cells. Cells were sorted and analyzed on a FACSTAR Plus™ (Becton Dickinson, San Jose, CA) equipped with two lasers, one emitting at 488 nm and a second (CR-599, Coherent, Palo Alto, CA) tuned to 600 nm to detect sulforhodamine fluorescence.

For 3 color analysis on the FACScan™, the above buffer was used except that 2% dialyzed BSA replaced the 2% FCS. Cells were stained with IgM isotype or EM16 mAbs and detected with the double layer of PE-labeled antibodies as described above. Biotinylated anti-CD38 (Leu-17, Becton Dickinson) was added at the same time as the PE-goat anti-rabbit IgG. After incubation and washing, FITC™-labeled anti-CD34 and streptavidin Red613 (Gibco, 1/50 dilution) were added and incubated for 30 minutes. Cells were washed a final time and cell pellet resuspended in buffer containing propidium iodide. 2000 FITC™ positive, low SSC events ($CD34^+$ cells) were collected and displayed as FL2 (EM16 or IgM isotype) versus FL3 ($CD38^+$ for moderately bright, or dead for extremely bright).

Sorting: Sort gates were established to collect cells that were sulforhodamine positive ($CD34^+$), propidium $iodide^{dim}$ (viable). These cells were further divided into subsets based upon the level of PE staining ($EM16^+$ and $EM16^-$).

Coexpression of CD34, Thy-1, and EM16, on rhodamine 123^{lo} cells from bone marrow: Cells were stained with rhodamine 123 (Molecular Probes, Eugene, OR) by incubating in buffer with rhodamine 123 at 0.1 Tg/mL for 30 minutes at 37°C, washing to remove excess rhodamine and then incubating cells in buffer without rhodamine for 30 minutes at 37°C to allow efflux. Subsequently, cells were stained at 4°C with EM16 or control IgM (detected with PE-labeled rabbit anti-mouse IgM, followed by PE-labeled goat anti-rabbit IgG, both from Zymed and diluted 1/100, FL2), anti-Thy-1 (GM201, detected with Texas Red-labeled goat anti-mouse IgG_1, FL5) and anti-CD34 (Tuk 3, Cy5-labeled FL4) and propidium iodide (FL3). Rhodamine staining is detected in the FL1 channel.

The results are shown in Figures 1-4. Figure 1 shows a FACScan™ analysis of bone marrow cells gated on $CD34^+Thy\text{-}1^+$ (Panel A), live, low side scatter events (Panel B), stained with an IgM isotype control (Panel C) or EM16 (Panel D) and rhodamine 123 (Panel D). The results show that EM16 stains a population containing nearly all $CD34^+Thy\text{-}1^+$cells, which are known to contain stem cells, including both $rhodamine^{lo}$ and $rhodamine^{hi}$ subsets. Figure 2 shows the staining of bone marrow cells with either Thy-1 or EM16 (Panels C and E, respectively), with isotype controls

(Panels B and D), of $CD34^+$ gated cells (Panel A). Staining with either Thy-1 or EM16 antibody is shown on the x-axis, and CD38 antibody staining is shown on the y-axis. The results show that EM16, like Thy-1, stains nearly all $CD34^+CD38^-$ cells, which is the population that includes stem cells.

Figure 3 shows the staining of EM16 and Thy-1 on $CD34^+$ gated cells. Panel A is CD34/Thy-1 staining alone. Panel B is with an IgM isotype control. Panel C is CD34/EM16 staining. The results show that EM16 stains more $CD34^+$ cells than does Thy-1, the $EM16^+$ including both $Thy\text{-}1^+$ and $Thy\text{-}1^-$ cells. Figure 4 compares EM16 versus CD34 staining (Panel D) to Thy-1 versus CD34 staining (Panel B) of bone marrow, with isotype controls (Panels A and C). EM16 binds to slightly more than half of the $CD34^+$ population, similar to Thy-1, and EM16 stains very few $CD34^-$ cells. On average, EM16 binds 50% of $CD34^+$ cells from bone marrow.

Sys1 coculture analysis: Sorted cell populations $CD34^+$, $CD34^+EM16^+$, $CD34^+EM16^-$, $CD34^+Thy\text{-}1^+$, $CD34^+Thy1^-$ were analyzed by limiting dilution analysis for cobblestone area forming cell frequency (CAFC) at 3–7 weeks of coculture on Sys1 stromal cells by limiting dilution analysis as described in Baum et al. (1991) *PNAS* 89:2804-2808, with the addition of human recombinant IL-6 (10 ng/ml) and LIF (20 ng/ml) to enhance the proliferation of adult bone marrow cells. The ability to give rise to both myeloid and B lymphoid progeny was determined after 6–7 weeks of coculture by staining with anti-CD19-FITC, anti-CD15-FITC and anti-CD33-PE (all from Becton Dickinson) and analyzing on the FACScan™. Results shown in Figure 5 show that the $CD34^+EM16^+$ population is highly enriched in CAFC frequency at weeks 3, 4 and 5, relative to the $CD34^+EM16^-$ population. Week 5 CAFC data is summarized in Table 1. Staining of week 5 cultures showed the presence of both lymphoid ($CD19^+$) and myeloid ($CD15^+$) cell development (data not shown).

SCID-hu thymus assay: Sorted cell populations were microinjected into depleted fetal thymic pieces and implanted under the kidney capsule of SCID-hu mice as described in Galy, et al. 1994 Blood 84:104–110. Six weeks after implantation, thymic pieces were recovered and analyzed for the presence of T cell progeny. Grafts

were scored as positive if ≥1% thymocytes were donor-derived. Data shown in Table 1 demonstrate that the $EM16^+$ population contains the T-cell potential.

SCID-hu bone assay: Sorted cell populations were injected into freshly irradiated fetal bone pieces that had been implanted subcutaneously into SCID-hu mice as described in Kyoizumi, et al. (1992) Blood 79:1704–1711. Eight weeks after injection the bone grafts were analyzed for myeloid and lymphoid progeny. Grafts were scored as positive for donor cell engraftment if ≥1% of the cells harvested from the graft were donor-derived. Results are shown in Table 1. Grafts reconstituted with $CD34^+EM16^+$ contained from 4–12% $CD33^+$ cells indicative of myeloid development and from 4–36% $CD19^+$ cells indicative of lymphoid development, and 1–8% $CD34^+$ cells indicative of primitiveness of the engrafting population.

Methylcellulose assay: Sorted cell populations were plated into methylcellulose cultures to determine colony forming cell activity as described in Brandt, et al. (1992) Blood 79:634–641. Added growth factors were kit ligand (100 ng/ml), erythropoietin (2 U/ml), GM-CSF (10 ng/ml) and IL-3 (10 ng/ml). The data in Table 1 show that the $EM16^+$ population contains nearly all day 14 CFU-GM and CFU-Mix, while both the $EM16^+$ and $EM16^-$ populations contain similar numbers of BFU-E.

Indirect immunofluorescent staining of cultured cell lines or fresh Peripheral Blood Leukocytes (PBL): The binding of EM16 mAbs to various cell lines before and after treatment with enzymes was detected using PE-labelled rabbit anti-mouse IgM (Zymed). (Table 3). The data show the EM16 marker is distinct from Thy-1, CD34 and other markers described on hematopoietic cells and hematopoietic stem cells. For determination of neuraminidase sensitivity of the epitopes, HEL or bone marrow cells were incubated at 10^7 cells/ml with 0-500 mU/ml of neuraminidase for 1 h at 37°C. For determination of glycoprotease sensitivity of the epitopes, cells were incubated at 10^7/ml in HBSS and a 1/10 dilution of O-sialoglycoprotein endopeptidase (CLE100, Accurate Chemical) was added. Cells were incubated at 37°C for 30 minutes. Cells were washed and subsequently tested for antibody binding by indirect immunofluorescence. Treatment of bone marrow cells with either neuraminidase or

glycoprotease does not diminish the binding of αEM16, showing that carbohydrate components are not a major aspect of the epitope recognized by αEM16.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

Table 1

			Functional Readouts of $CD34^+Lin^-$ Cell Populations Subsetted with EM16 or Thy-1 mAbs						# Grafts Repopulated per # Injected	
Antibody	Tissue	Sort Purity	% of CD34+ cells also positive for EM16 or Thy	Population Assayed	CAFC frequency week 5	CFC/10^4 cells (day 14) BFU-E/CFU-GM/CFU-Mix	HPP/10^5 cells (day 24)	Cumulative CFU-GM	SCID-hu bone	SCID-hu thymus
EM16										
	9041	2-7% cross contamination	79%	EM16+	1/23	12325 / 1925 / 175				
				EM16-	1/336	1800 / 225 / 0				
	10362	4-15% cross contamination	38%	EM16+	1/22	1590 / 2200 / 1050				3/5
				EM16-	1/28	7975 / 1825 / 500				0/5
	10856	2-3% cross contamination	83%	EM16+	1/67	3400 / 2900 / 200	1900	43	4/6	
				EM16-	1/850	1600 / 100 / 0	0	0	4/6	
	12619	2-4% cross contam.	80%	EM16+	1/22	2200 / 3200 / 100	1700	47.5	mice died, rad. poison	
				EM16-	1/473	3700 / 0 / 0	100	1		
Average of EM16				EM16+	1/27	4879 / 2556 / 381	1800			
				EM16-	1/95	3769 / 538 / 125	50			

Table 1 (cont'd)

Antibody	Tissue	Sort Purity	% of CD34+ cells also positive for EM16 or Thy	Population Assayed	CAFC frequency week 5	CFC/10+ cells (day 14) BFU-E/CFU-GM/CFU-Mix	HPP/10^5 cells (day 24)	Cumulative CFU-GM	# grafts repopulated / SCID-hu bone	# injected SCID-hu thymus
Thy-1 (GM201)										
	9041	2%	50%	Thy+	1/26	1125 / 925 / 75				
		cross contam.		Thy-	1/1084	10600 / 250 / 25				
	10856	1-11%	45%	Thy+	1/64	400 / 2900 / 0	1900	38.5	4/4	
		cross contam.		Thy-	1/81	10500 / 9500 / 400	5000	19.5	not done	
	12619	2-10%	45%	Thy+	1/21	800 / 3300 / 200	3200	80.5	mice died, rad. poison	
		cross contam.		Thy-	1/74	2000 / 1100 / 100	500	9		
Average of Thy				Thy+	1/29	775 / 2375 / 92	2550			
				Thy-	1/112	7700 / 3617 / 175	2750			
Cumulative CFU-GM is the number of CFU-GM arising from 8 replicate 50 cell cultures (on Sys 1)which were transferred in duplicate to methycellulose culture at weekly intervals for 4 weeks and subsequently scored at day 14 for CFU-GM.										

Table 2

Binding of EM16 to apheresed mobilized peripheral blood

TISSUE	DISEASE	% of CD34+ cells that are:		% of CD34$^-$ cells that are:	
		EM16+	THY+	EM16+	THY+
10680	NHL	77	38	2	ND
7259	NHL	13	24	2	0
11449	NHL	46	39	4	0
10772	NHL	64	60	6	2
8947	MM	52	45	24	4
8800	MM	66	38	0	3
95162	MM	52	85	ND	ND
10030	BC	87	44	6	0
11367	BC	86	43	0	0
7053	BC	56	ND	ND	ND
11136	Normal	73	35	ND	ND
11200	Normal	76	56	ND	ND
	RANGE	13–87	24–85	0–24	0-4

Legend:
NHL=Non-Hodgkin's Lymphoma
MM=Multiple myeloma
BC=Breast Cancer
ND=Not Determined

Table 3

Cell and Cell Line Staining by various mAbs, % positive

	Cell Line														
mAb	KG1a	KG1	Jurkat	N417	HEL	Daudi	Raji	HL60	K562	U937	TF1	MCF7	T47D	HUVEC	HUVEC+ IL-1β (4h)	pBL
CD34	99	99	40	<5	24	<5	<5	<5	<5	<5	65			5	6	<3
Thy-1	<5	<5	100	<5	98	<5	<5	<5	<5	<5		<5	10	<5	<5	3
EM16	<5	<5	<5	50	80	10	7	<5	<5	<5	90	<5	<5	<5	6	<3
CD31	76		90	<5	90	16	<5		45	100	94	<5	<5	100	100	55

**Blank spaces in Table indicates cells were not tested for that antibody, not that it was not reactive.

CLAIMS

1. A method of obtaining a composition substantially enriched in hematopoietic stem cells comprising the steps of:

combining a cell population containing hematopoietic stem cells with an antibody that specifically recognizes and binds to EM16 under conditions which allow the antibody to specifically bind to EM16; and

isolating the cells recognized by the antibody to obtain a composition substantially enriched in hematopoietic stem cells.

2. The method according to claim 1, further comprising the step of selecting for cells expressing at least one additional marker associated with hematopoietic stem cells.

3. The method according to claim 2, wherein the additional marker is CD34 or Thy-1.

4. The method according to any one of claims 1 to 3, further comprising the step of selecting for cells that lack at least one lineage specific (LIN^-) marker.

5. The method according to claim 4, wherein the lineage specific marker is selected from the group consisting of CD14, CD15, CD38, HLA-DR, CD71 and CD33.

6. The method according to claim 4, wherein the lineage specific marker is selected from the group consisting of CD2, CD16, CD19, CD20 and glycophorin A.

7. A composition comprising a substantially enriched population of human hematopoietic stem cells, wherein the cells express the EM16 marker.

8. The composition according to claim 7, wherein the cells are further characterized as having at least one additional marker associated with hematopoietic stem cells.

9. The composition according to claim 8, wherein the additional marker is CD34 or Thy-1.

10. The composition according to any one of claims 7 to 9, wherein the cells are characterized for lacking at least one lineage specific (LIN^-) marker.

11. The composition according to claim 10, wherein the lineage specific marker or markers is or are selected from the group consisting of CD14, CD15, CD38, HLA-DR, CD71 and CD33.

12. The composition according to claim 10, wherein the lineage specific marker or markers is or are selected from the group consisting of CD2, CD16, CD19, CD20 and glycophorin A.

13. A method of determining the stem cell content in a sample of hematopoietic cells comprising the steps of:

a) combining a mixture of hematopoietic cells with an antibody that recognizes and binds to EM16 under conditions which allow the antibody to specifically bind to EM16; and

b) quantitating the cells recognized by the antibody, thereby determining the stem cell content.

14. The method according to claim 13, further comprising the step of selecting the cells for expression of at least one additional marker associated with hematopoietic stem cells.

15. The method according to claim 14, wherein the additional marker is CD34 or Thy-1.

16. The method according to any one of claims 13 to 15, further comprising the step of selecting the cells for lack of expression of at least one lineage specific (LIN^-) marker.

17. The method according to claim 16, wherein the lineage specific marker or markers is or are selected from the group consisting of CD14, CD15, CD38, HLA-DR, CD71 and CD33.

18. The method according to claim 16, wherein the lineage specific marker or markers is or are selected from the group consisting of CD2, CD16, CD19, CD20 and glycophorin A.

19. An antibody that specifically recognizes and binds EM16.

20. The antibody of claim 19, wherein the antibody is a monoclonal antibody.

21. A biological active fragment of the antibody of either claim 19 or 20.

22. Antibody according to either claim 19 or 20 which is produced by HB-12100 or its progeny.

23. Use of the antibody as claimed in any one of claims 19, 20 or 22 in a method of isolation or determining the stem cell content of a stem cell composition.

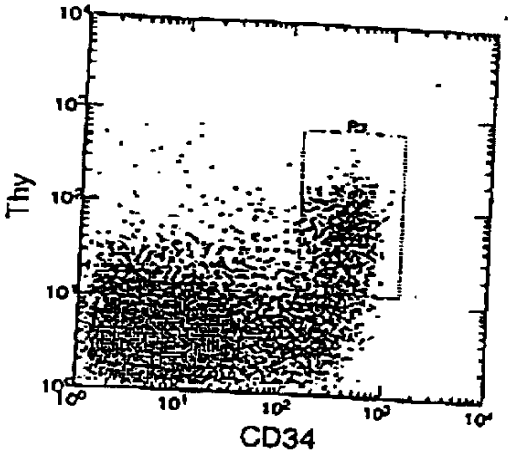
24. A cell line that produces the antibody of claim 19.

25. A cell line as claimed in claim 24 which is HB-12100.

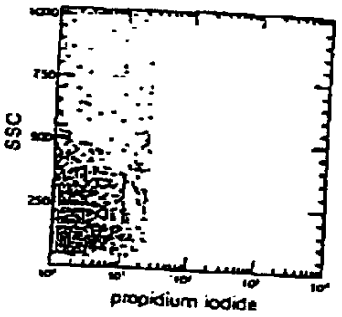
26. A composition obtainable according to a method as claimed in any one of claims 1 to 6.

27. A method of treatment of a disease or amelioration of symptoms associated with a disease, amenable to gene transfer into a composition as claimed in any one of claims 7 to 12, wherein the composition is administered to a subject to be treated.

28. Use of an antibody which specifically recognizes and binds EM16 in a method of obtaining a composition substantially enriched in hematopoietic stem cells.



A



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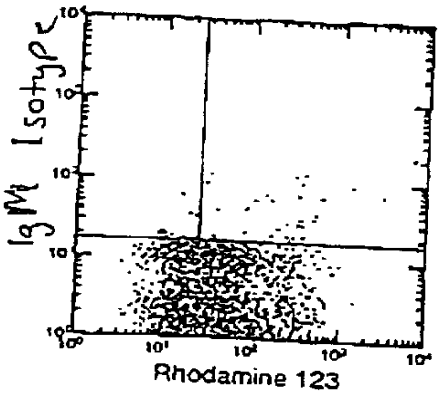
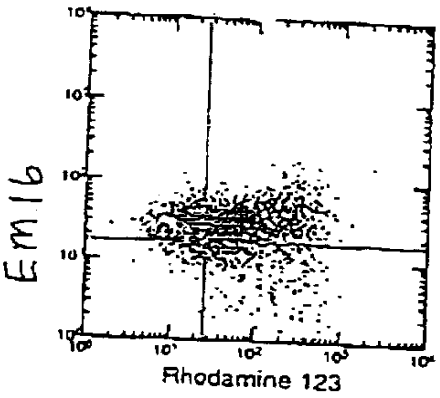


C



D

FIGURE 1

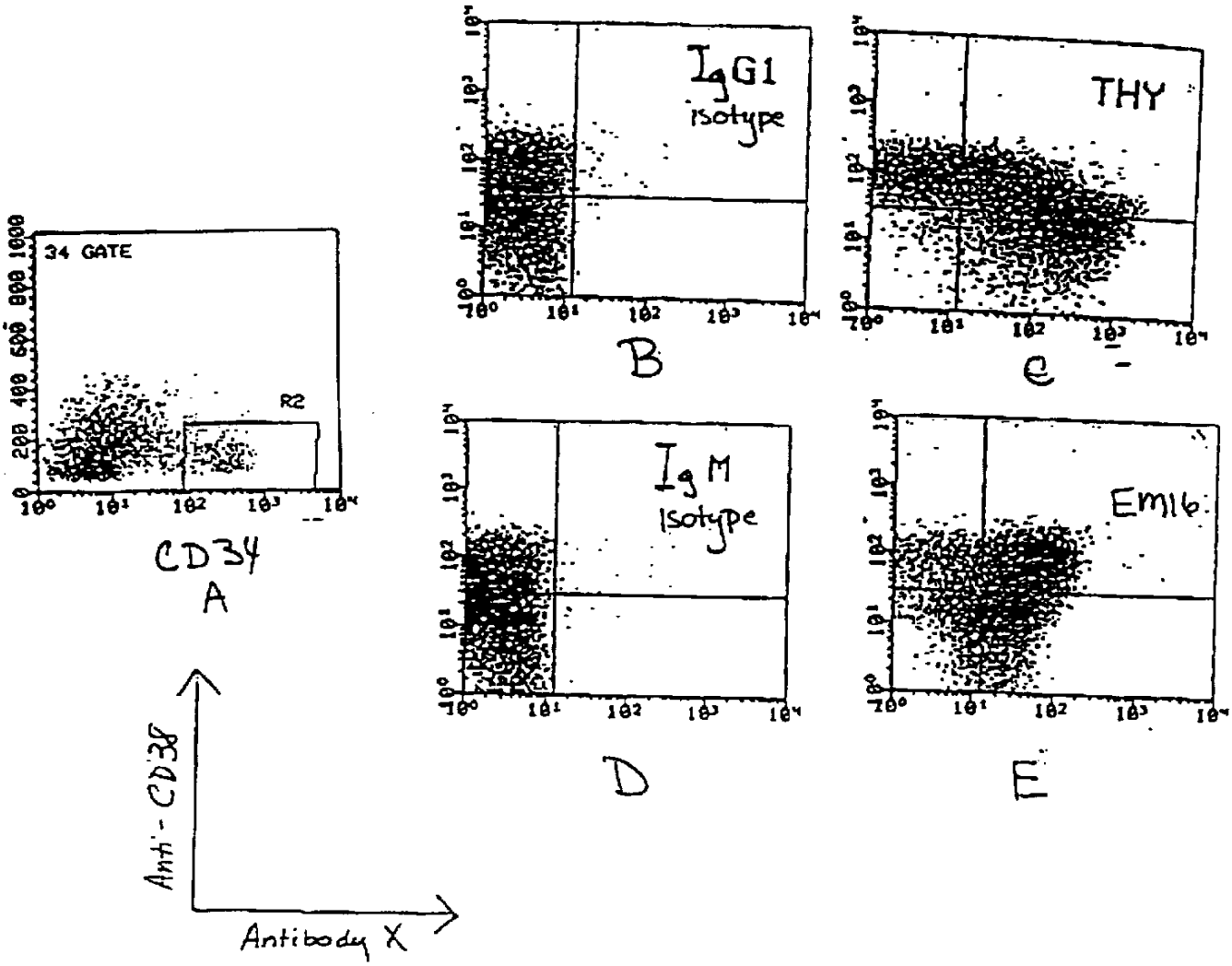

34 GATE
R2
CD34
A
IgG1
isotype
B
THY
C
IgM
isotype
D
Em16
E
Anti-CD38
Antibody X

FIGURE 2

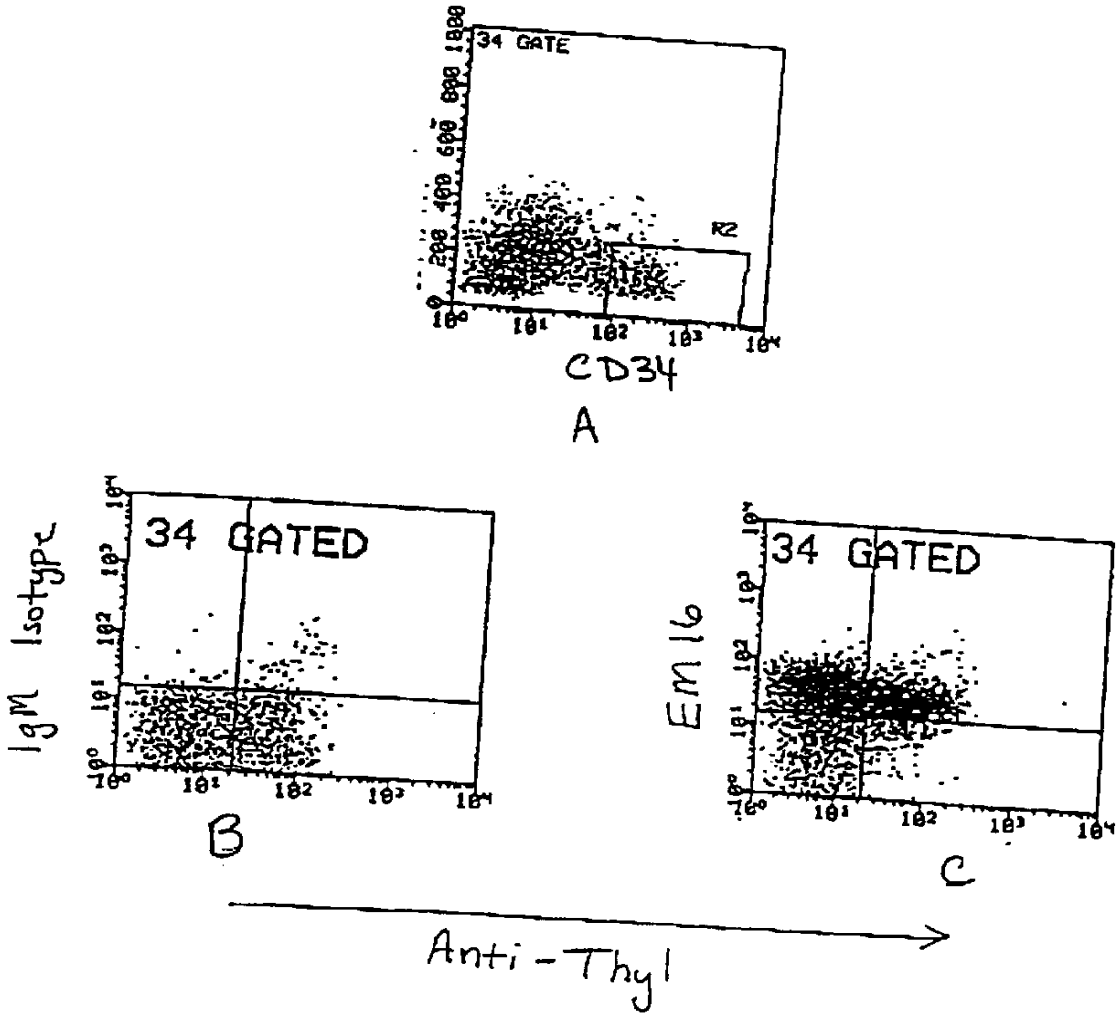

34 GATE
R2
CD34
A
34 GATED
IgM Isotype
B
34 GATED
EM16
C
Anti-Thy1

FIGURE 3

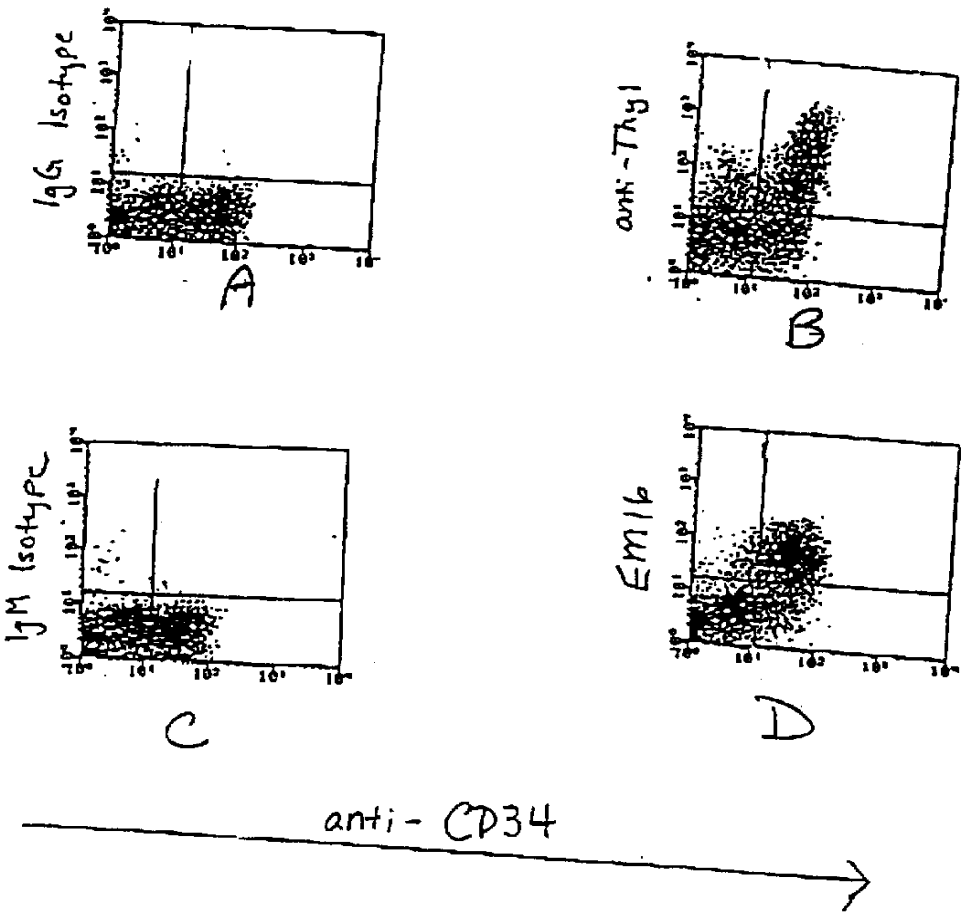


FIGURE 4

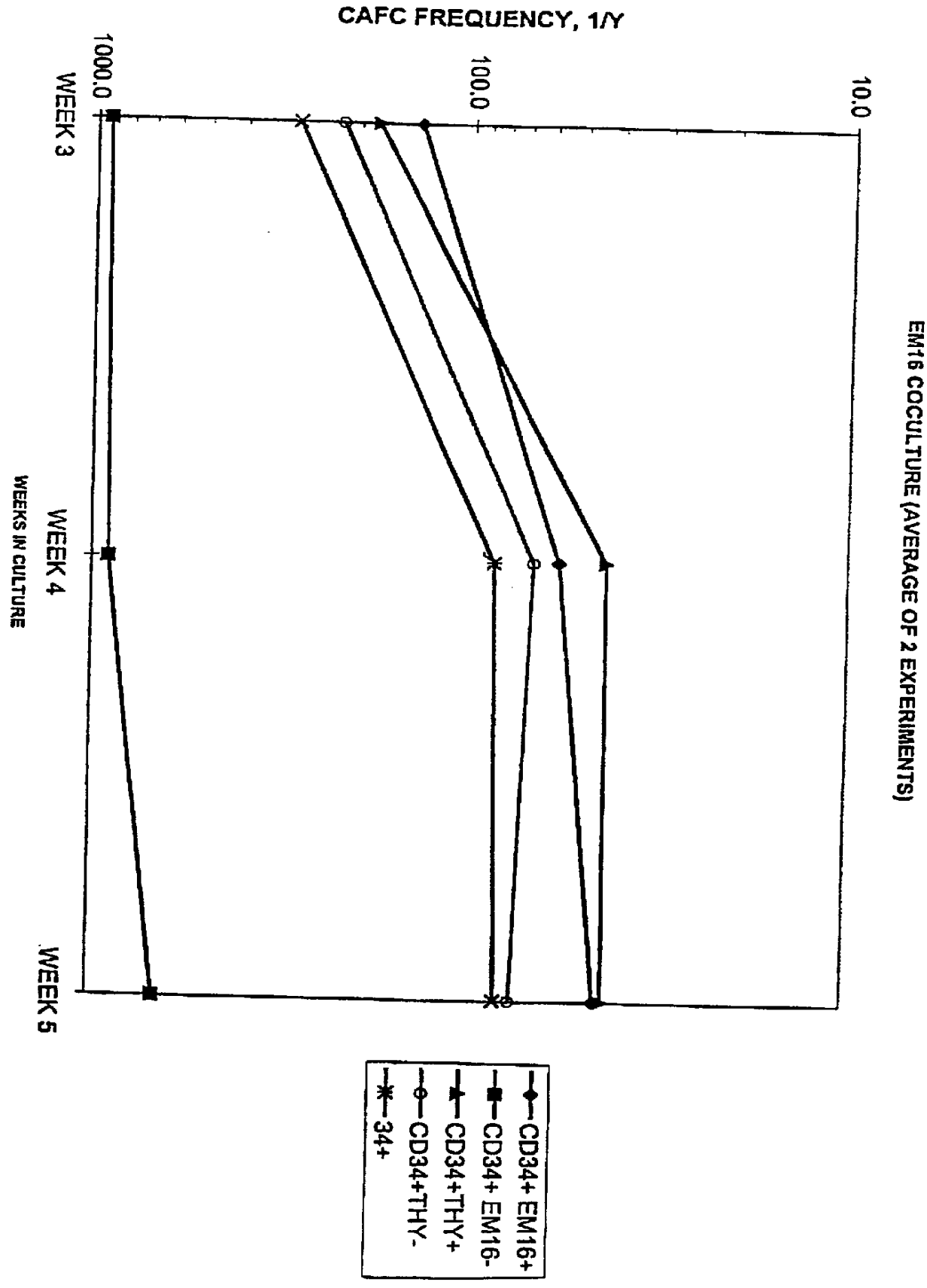

CAFC FREQUENCY, 1/Y
1000.0
100.0
10.0
WEEK 3
WEEK 4
WEEK 5
WEEKS IN CULTURE
EM16 COCULTURE (AVERAGE OF 2 EXPERIMENTS)
CD34+ EM16+
CD34+ EM16-
CD34+THY+
CD34+THY-
34+

Figure 5

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 97/03422

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N5/08 C07K16/28 A61K35/28 G01N33/577

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 03693 A (SYSTEMIX, INC.) 9 February 1995 see the whole document	1-28
A	WO 96 15229 A (SYSTEMIX, INC.) 23 May 1996 see the whole document	1-28
P,A	WO 96 40874 A (SANDOZ LTD.; SYSTEMIX, INC.) 19 December 1996 see the whole document	1-28
P,A	WO 96 40875 A (SANDOZ LTD.; SYSTEMIX, INC.) 19 December 1996 see the whole document	1-28

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
13 October 1997	29. 10. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte onal Application No
PCI/EP 97/03422

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9503693 A	09-02-95	AU 7476694 A	28-02-95
		CA 2168348 A	09-02-95
		EP 0711111 A	15-05-96
		JP 9502167 T	04-03-97
WO 9615229 A	23-05-96	AU 3752795 A	06-06-96
		EP 0787181 A	06-08-97
WO 9640874 A	19-12-96	AU 6223196 A	30-12-96
WO 9640875 A	19-12-96	AU 6223296 A	30-12-96